Assay Validation and Clinical Performance of Chronic Inflammatory and Chemokine Biomarkers of NASH Fibrosis

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Introduction

Nonalcoholic steatohepatitis (NASH) is a chronic liver disease that can lead to cirrhosis, liver transplant, and even hepatocellular carcinoma. Liver biopsy remains the reference standard for disease diagnosis.

However, development of noninvasive soluble biomarkers of NASH are of great importance for early detection and for drug development. While fibrosis panels based on clinical chemistry laboratory results such as FIB4, a composite index of AST, ALT, platelet count and age, can discriminate between disease stages, its diagnostic performance is inadequate.

To qualify new biomarkers, the Food and Drug Administration (FDA) developed the Biomarker Qualification Program to establish standards for validating the analytical measurement and clinical utility of a biomarker for a specific context of use (COU). Therefore, investigation of novel, noninvasive and soluble biomarkers with robust analytical and clinical validation for diagnosing NASH and fibrosis severity is required.

In this study, we developed and validated assays for a set of seven pro-inflammatory cytokine and macrophage recruitment chemokine biomarkers (IL-6, TNF-α, MCP-1, MIP-1β, eotaxin, VCAM-1 and CRP) and clinically examined their performance as a biomarker in biopsy-confirmed NASH patients.

Methods

The test population consisted of 52 patients with biopsy-proven NAFLD/NASH who enrolled in an on-going tissue and serum repository at Brooke Army Medical Center, TX. The study protocol was approved by the Brooke Army Medical Center ethics review board and conducted according to the principles of the Declaration of Helsinki. All subjects underwent a liver biopsy. Participants were categorized based on hepatic fibrosis stage; no fibrosis (F0), mild fibrosis (F1-F2) and advanced fibrosis (F3-F4).

The serum levels of IL-6 and TNF-α were measured using the human V-plex Proinflammatory Panel 2 (MSD, Kenilworth, NJ). MCP-1, MIP-1β, and eotaxin were measured using the human V-plex Chemokine Panel 1 (MSD). The serum levels of VCAM-1 and CRP were measured using the human V-plex Vascular Injury Panel 2 (MSD). All assays were modified appropriately to meet the FDA bioanalytical guidance and industry best practices. For all assays, three quality control (QC) samples were prepared in-house by spiking recombinant protein in an appropriate surrogate matrix to span the entire standard curve range. Assays were validated for accuracy and precision before sample analysis.

The following adaptations of the commercial biomarker kits were made during method development for drug development use:

- The kits contained only 4 standards in the quantification range. Since 6 are required for drug development assays, 2 additional standards were added.
- The QC samples from the kit were only certified to an acceptable range. New QC's were made in-house with an exact known concentration.

Results

The dynamic range of the biomarker assays were 2.45 pg/mL – 444 pg/mL for IL-6; 69.2 pg/mL – 108,000 pg/mL for CRP; 0.79 pg/mL – 211 pg/mL for TNFα; 8.28 pg/mL—1060 pg/mL for MCP-1, 17.5 pg/mL – 2240 pg/mL for MIP-1β; 56.8 pg/mL – 3640 pg/mL for eotaxin; and 90.8 pg/mL – 284,000 pg/mL for VCAM-1. The endogenous concentrations of analytes in serum for all subjects were within these analytical ranges.

Fig 1. Assay validation of commercial biomarker kits for drug development use. (A) IL-6, (B) CRP, (C) TNFα, (D) MCP-1, (E) MIP-1β, (F) Eotaxin, (G) VCAM-1. Dotted lined indicates 95% limits of agreement. Mean age of the entire cohort was 52.1±10.5 years and 62% men.

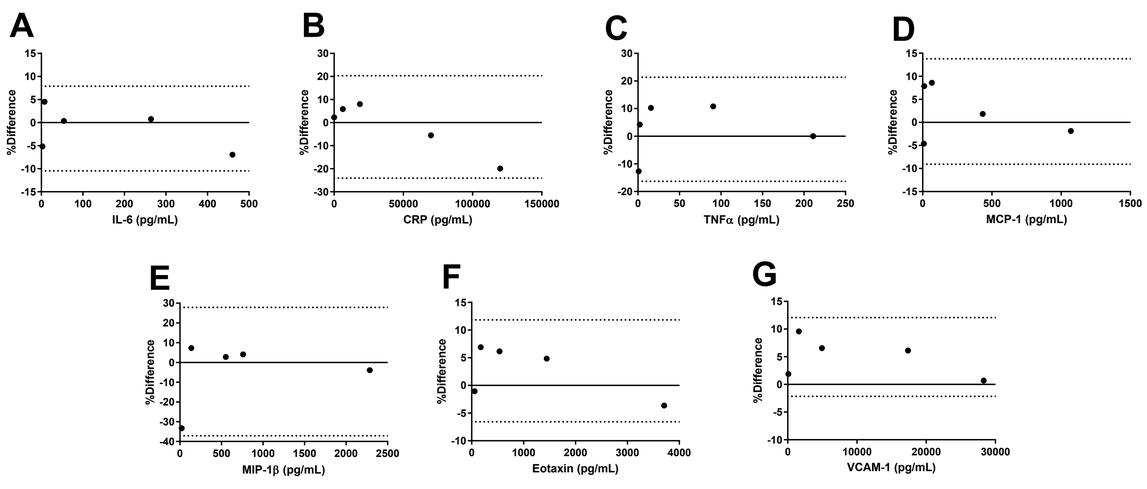


Table 1. Subject Characteristics.

Parameter	No Fibrosis (n=20)	Mild Fibrosis (n=20)	Advanced Fibrosis (n=12)
Sex (%)			
Male	65	55	67
Female	35	45	33
Age (years)	47.7±11.5	51.4±8.9	60.9±4.6
Weight (lbs)	214.6±40.4	212.1±48.6	210.3±55.0
BMI (kg/m^2)	33.4±5.6	33.4±4.8	32.2±5.8
AST (U/L)	37.4±21.0	55.7±32.6	58.5±47.2
ALT (U/L)	60.1±36.8	84.2±47.7	62.0±46.9
Platelet count (x10^9)	250.8±55.5	247.3±70.0**	183.5±115.5$^{**,\#\#}$
Triglyceride (mg/dL) †	159.8±85.5	172.7±124.5	105.3±26.4$^{*,\#\#}$
LDL (mg/dL)	97.6±25.5	92.3±31.3	89.8±19.7
HDL (mg/dL)	40.4±12.6	46.5±13.3	44.1±14.5
Glucose (mg/dL) †	95.7±20.2	121.2±51.7	110.4±37.0
Insulin (uU/ml)	23.00±21.04	16.58±11.11	22.04±15.23
FIB4	1.19+0.56	1.57+0.64	2.97+1.64$^{***,\#\#\#}$
Liver Grade	0.50±0.51	1.45±0.51***	1.83±0.39***

† n=11 for advanced fibrosis cohort. *p<0.05, ** p<0.01, ***p<0.001 vs No Fibrosis, ##p<0.01, ###p<0.001 vs Mild Fibrosis

Fig 2. Inflammatory cytokines analyzed by NASH fibrosis severity.
Serum IL-6 was significantly elevated in patients with advanced liver fibrosis compared to no and mild fibrosis patients. (A) IL-6, (B) CRP, and (C) TNF-α. **p<0.01 vs Advanced Fibrosis group.

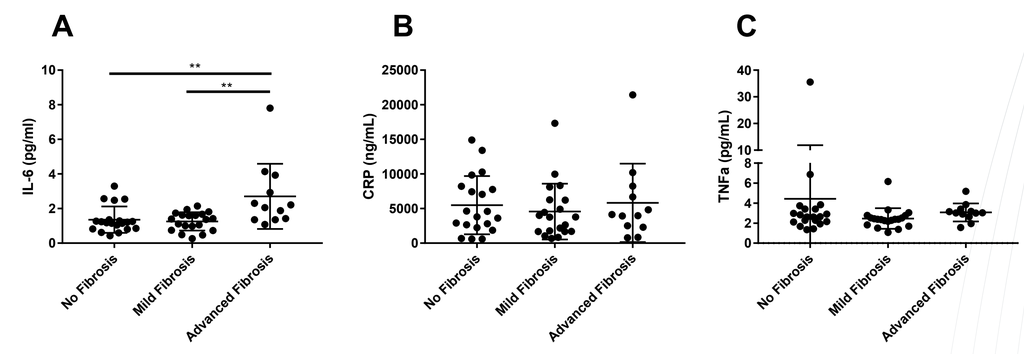


Fig 3. Chemokines and VCAM-1 analyzed by NASH fibrosis severity.
Serum VCAM-1 was significantly elevated in patients with advanced liver fibrosis compared to no and mild fibrosis patients. Chemokines were not significantly altered with increasing NASH fibrosis. (A) MCP-1, (B) MIP-1β, (C) Eotaxin and (D) VCAM-1. *p<0.05, **p<0.01 vs Advanced Fibrosis group.

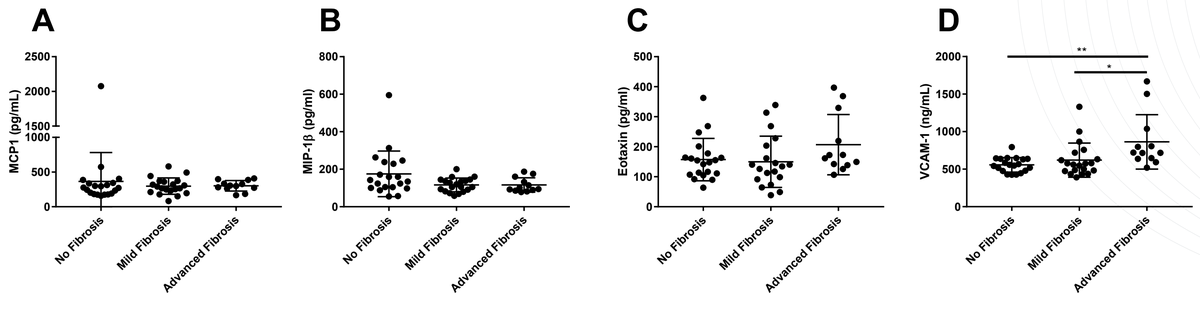
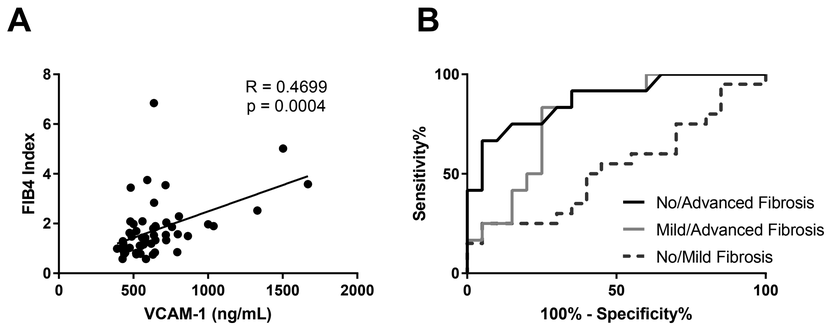


Fig 4. VCAM-1 demonstrates good performance in distinguishing advanced fibrosis in NASH.
(A) VCAM-1 correlates with FIB4 values. (B) ROC curves demonstrating performance of VCAM-1 to distinguish fibrosis severity in NASH. AUROC of 0.87, 0.79, 0.53 for no/advanced, mild/advanced, and no/mild fibrosis severity respectively.



Conclusion

- In a single study comparison, the role of several cytokines and chemokines as biomarkers for NASH fibrosis were examined.
- Analytical validation is required to satisfy regulatory guidance for biomarker use in a clinical trial. To this end, we demonstrated good accuracy and assay robustness for VCAM-1 as well as clinical value.
- The VCAM-1 assay demonstrated robust accuracy and precision, and VCAM-1 outperformed IL-6, CRP, TNFα and the chemokines MCP-1, MIP-1β, and eotaxin as a biomarker of advanced fibrosis in NASH.
- Addition of biomarkers such as IL-6 and VCAM-1 to panels may yield increased sensitivity and specificity for staging of NASH.

References

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